

Excretions/Secretions from Bacteria-Pretreated Maggot Are More Effective against *Pseudomonas aeruginosa* Biofilms

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Abstract

Background: Sterile larvae—maggots of the green bottle blowfly *Lucilia sericata* are employed as a treatment tool for various types of chronic wounds. Previous studies reported that excretions/secretions (ES) of the sterile larvae could prevent and remove the biofilms of various species of bacteria. In the present study we assessed the effect of ES from the larvae pretreated with *Pseudomonas aeruginosa* on the bacteria biofilms.

Methods and Findings: We investigated the effects of ES from the maggot pretreated with *P. aeruginosa* on the biofilms using microtitre plate assays and on bactericidal effect using the colony-forming unit (CFU) assay. The results showed that only 30 µg of the ES from the pretreated maggots could prevent and degrade the biofilm of *P. aeruginosa*. However, the CFU count of *P. aeruginosa* was not decrease when compared to the ES from non pretreated maggots in this study condition. It is suggested that the ES from the pretreated maggot was more effective against biofilm of *P. aeruginosa* than sterile maggot ES.

Conclusions: Our results showed that the maggot ES, especially the bacteria-pretreated larva ES may provide a new insight into the treatment tool of the bacterial biofilms.

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Introduction

As the population ages, the number of patients suffering from chronic wounds attributable to diseases such as diabetes mellitus and peripheral vascular disease is on the rise [1]. The healing process is often complicated by bacterial infections on the wound surface [2], especially when the bacteria are residing in biofilms [3]. Biofilm bacteria exhibit altered growth characteristics and gene expression profiles as compared with those planktonic in the environment [4]. An important consequence following biofilm formation is that the bacteria are protected against the actions of antibiotics and the effector molecules of the immune system [5,6].

Sterile larvae of the green bottle blowfly *Lucilia sericata* are used as a treatment tool for various types of chronic wounds [7]. In clinical practice, fast healing of infected wounds by means of maggot debridement therapy (MDT) in combination with antibiotics has been observed [8]. The molecules involved in these actions are believed to be contained in the excretions/secretions (ES) of maggots. Recently, researches showed that Sterile Maggot ES could effectively perform against biofilms of *S. aureus* and *P. aeruginosa* [9,10,11]. However, in MDT, once the sterile larvae are applied to an infected wound, they are no longer

germ-free, becoming infected state without physical injury. Previous studies have described that the antibacterial capacities of the infected larvae were better than those of sterile larvae [12]. Since modulation of bacterial biofilms will have a major impact on the healing process of infected wounds, we assessed the effect of ES from *P. aeruginosa*-infected maggot (pretreated ES) on the formation of biofilms and on the disruption of established biofilms of the bacteria.

Materials and Methods

Maggots and maggot ES

ES of sterile second-and or third-instar larvae of *L. sericata* from our own laboratory was collected as described by van der Plas et al [13]. In short, 500 larvae were incubated in physiological saline for 60 min at 37°C, in darkness.

ES of pretreated larvae was collected as described by Basset et al [14]. Sterile third-instar larvae were incubated with different amounts of bacteria for 2 hours and then the larval ES was collected and centrifuged at 13,000 × g for 10 minutes at 4°C to remove particulate material. Then, the supernatant was filtered with 0.22 µm filtration membrane and stored at –20°C or for use.

The concentration of ES protein was determined using the Pierce BCA Protein Assay Kit (Pierce, USA) according to manufacturer's instructions.

Bacterial strains and growth conditions

The strain of *P. aeruginosa* was isolated from patients of our department from infected wounds, and then grown in 3% Tryptone Soya Broth (TSB) at 37°C under vigorous shaking.

Biofilm assay

Biofilm formation of *P. aeruginosa* in 96-well polyvinyl chloride plates was conducted as described by van der Plas et al [9]. In short, Bacteria from over night cultures were diluted with medium in 1:100 and 5 µL aliquots of these bacterial suspensions were added to each well of the 96-well flat-bottomed microtiter plate, which contains 130 µL of the medium with or without ES. After 24 h incubation, planktonic cells were removed and the wells were washed with tap water. Subsequently, biofilms were exposed to a 1% crystal violet solution for 15 min, washed and then incubated in absolute ethanol for 15 min to extract the crystal violet retained by the cells. Next, this solution was measured at a wavelength of 590 nm to quantify the formed biofilm.

Measurement of bactericidal effect *in vitro*

To determine the bactericidal effect of ES on planktonic bacteria, *P. aeruginosa* was incubated at 37°C with different concentrations of ES, or with sterile physiological saline as control. The aliquots of the samples were diluted with PBS after incubation for 24 h. Subsequently, 2 µL of the diluted solution were spread onto tryptone soya agar (TSA). After overnight incubation at 37°C, the number of colonies was counted manually.

Microscopic analysis

Scanning electron microscope and bright field microscope were used for visualizing the biofilms in the absence or presence of pretreated larval ES in the culture medium.

Statistical analysis

All statistical analyses were operated by GraphPad Prism software. One-way ANOVA and two-tailed Student t-tests were used in our statistical analysis, and SNK method was used for multiple comparisons. A P-value < 0.05 was considered as statistically significant.

Results

Effect of pretreated ES on biofilms formation

To find out whether pretreated ES can prevent biofilm formation, the bacteria-infected larva ES and bacterial suspensions were added to each well and then incubated for 24 h. The amount of biofilm was quantified by measuring the optical density. The result revealed that the amount of *P. aeruginosa* biofilm was CFU number dependently reduced (data not shown) and that in the 1×10^6 CFU bacteria-pretreated group the biofilm amount was significantly lower than in the sterile group (Figure 1).

Effect of pretreated ES on established biofilm

To investigate the effect of pretreated ES on established biofilms, we fed the sterile maggot with different amounts of bacteria. The larval extracts were collected after the set periods of incubation with bacteria or PBS, and then the ES was added to the well with *P. aeruginosa* biofilm. The result showed that the amount of *P. aeruginosa* biofilm in the 1×10^6 CFU pretreated group was

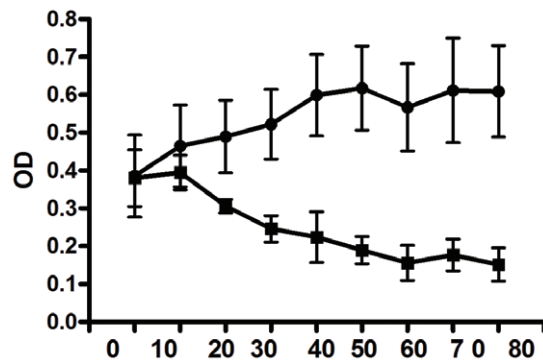


Figure 1. Effect of pretreated ES on biofilms formation. Bacterial suspensions were added to each well with pretreated ES or sterile ES. After 24 h incubation, biofilms were exposed to a 1% crystal violet solution, and the amount of biofilm was measured by its A590. For 20 µg on, all values are significantly ($p < 0.05$) different from these for biofilms without pretreated (●- nonpretreated group, ■- pretreated with 10^6 cfu/ml). doi:10.1371/journal.pone.0049815.g001

significantly lower than that in the PBS group (Figure 2a). Furthermore, after adding 30–80 µg of 1×10^6 CFU/ml pretreated ES, the *P. aeruginosa* biofilm breakdown was dose-dependently enhanced (Figure 2b).

The effect of pretreated ES was also examined on *P. aeruginosa* biofilm by light microscope and scanning electron microscope. It is demonstrated that the result was similar to that showing in Figure 1a. Both means after the pretreated ES treatment, the structure of *P. aeruginosa* biofilm was disrupted (Figure 2c).

Effect of pretreated ES on *P. aeruginosa* growth

It is reported that sterile larva ES may have bactericidal activities against Gram-positive and Gram-negative bacteria [15,16], we determined the effect of the pretreated ES on the number of viable biofilm-associated *P. aeruginosa* in our experiments. The result demonstrated that using the current doses and conditions maggot ES did not reduce the total number of bacteria in the wells (Figure 3).

Discussion

Bacteria within chronic wounds often reside in biofilms, which protect bacteria against the actions of antibiotics [4]. Previous reports showed that sterile maggot ES could be effective against biofilms of *P. aeruginosa* [9]. However, in clinical treatment, when sterile larvae are put of wound surface during MDT, they contact pathogenic bacteria and become non-sterile. Furthermore, the maggots of *Lucilia sericata* are successfully used as a treatment for infected wounds [17]. It is suggested that infected environment might not influence the efficiency of maggot ES against biofilms.

In this study, we demonstrated that ES from bacteria-pretreated larvae could also prevent and break down biofilm on *P. aeruginosa*. This conclusion is based on the following observations. First, only 30 µg pretreated maggot ES could break down established biofilms. Although a previous study showed that sterile maggot ES was effective against biofilms of *P. aeruginosa* [9], the pretreated maggot ES was considered to better reflect the context of an actual clinical wound [7]. Our results demonstrated that bacterial pretreatment of sterile larvae resulted in a dose-dependent increase in disrupting established biofilms. Secondly, the effect of 20 µg pretreated maggot ES, which could prevent biofilm formation is similar to that of sterile maggot ES [9]. Thus, the infection model

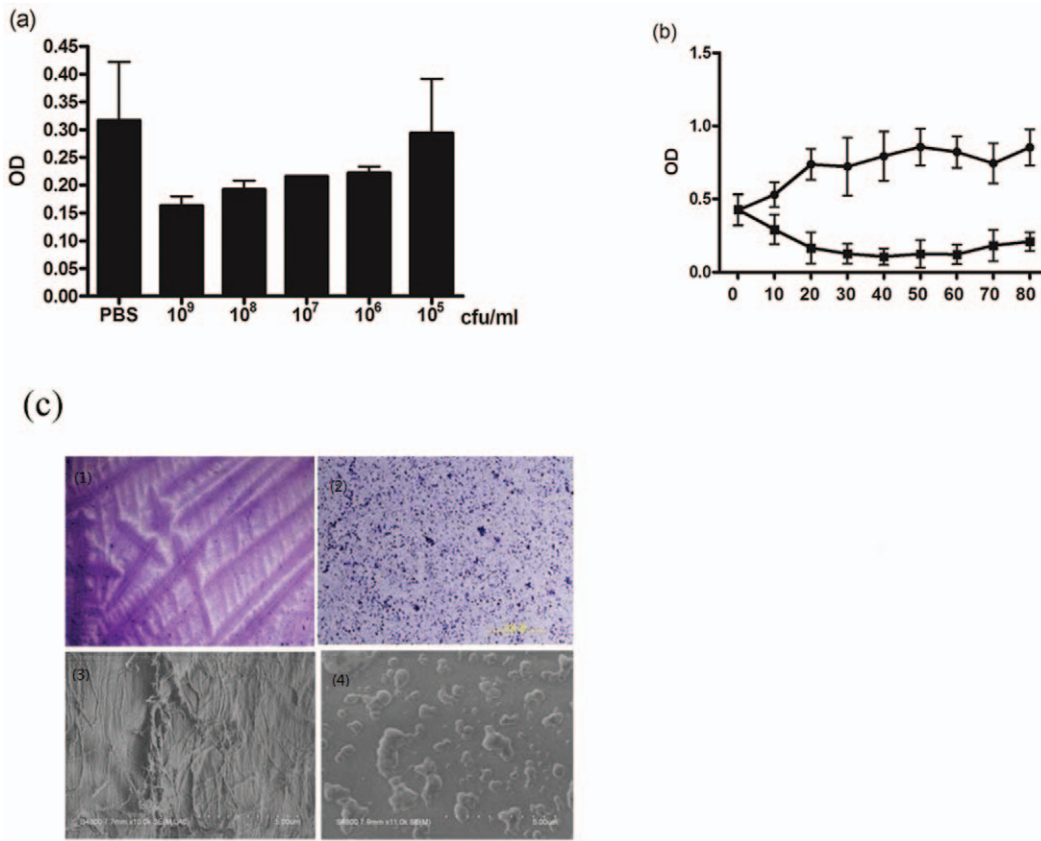


Figure 2. Effect of pretreated ES on established *P.aeruginosa* biofilm. (a) ES from different CFU of *P. aeruginosa* fed sterile larva, then added to the well which contained *P.aeruginosa* biofilm, 24 h incubation, biofilms were exposed to a 1% crystal violet solution, and the amount of biofilm was measured by its A590. (b) Different amounts of ES from 1×10⁶ CFU of *P. aeruginosa* fed sterile larva were added to the well which contained *P.aeruginosa* biofilm, 24 h incubation, biofilms were exposed to a 1% crystal violet solution, and the amount of biofilm was measured by its A590. For 30 μg on, all values are significantly ($p < 0.05$) different from these for biofilms without pretreated. (●) nonpretreated group, (■) pretreated with 10⁶ cfu/ml (c) Light microscopic and scanning electron microscopic graphs of *P.aeruginosa* biofilm with or without infected ES. (c-1) Light microscopic graph of *P.aeruginosa* biofilm; (c-2) Light microscopic graph of *P.aeruginosa* biofilm which treated with infected ES ; (c-3) Scanning electron microscopic graphs of *P.aeruginosa* biofilm ;(c-4) Scanning electron microscopic graphs of *P.aeruginosa* biofilm which treated with infected ES.

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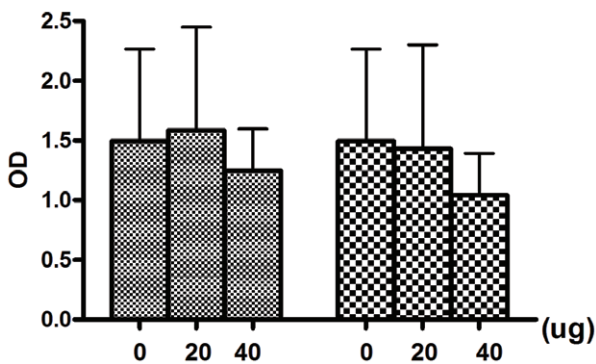


Figure 3. Antimicrobial activity of maggot ES against *P. aeruginosa*. The sterile larval were pretreated with 10⁶ cfu/mL *P. aeruginosa* or not, then 20 μg and 40 μg infected ES or sterile ES were added to each bacteria well respectively, 24 h later, the number of colony-forming unit was assayed. (■) nonpretreated group, (■) pretreated with 10⁶ cfu/ml).

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is very similar to the clinical wound context in MDT and will be a powerful tool to study the activities of *L. sericata* larvae in MDT.

However, the antibacterial activity against *P. aeruginosa* was not detected in pretreated larva ES, which was pretreated with 1×10⁶ bacteria. Previously published studies indicated that the antibacterial activities induced by *S. aureus* and *P. aeruginosa* were effective against *S. aureus*, but not against *P. aeruginosa* [12,15,18]. Moreover, previous clinical studies showed that MDT was more effective in Gram-positive infected wounds than in Gram-negative infected ones [19,20]. It is clear that maggot continuously secrete its product in wounds, but in our experiment we only added ES once to bacteria suspension. Therefore, It is presumed that more *P. aeruginosa* suspension would be needed to activate larval immune systems in future study.

Conclusions

This investigation demonstrated that the bacteria-pretreated ES has a capacity to inhibit biofilm formation and break down existing biofilm more effectively. Although further studies are needed, these results suggest that bacteria-infected larva may induce new products to survive in such a harmful environment which is very similar to the clinical context in MDT. Further

investigation would be needed to identify the bioactive compounds of infected larva ES, which may lead to better understanding the mechanisms of MDT [21].

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Author Contributions

Conceived and designed the experiments: A-PW K-CJ. Performed the experiments: K-CJ X-JS WW LL Y-CC NL J-HY D-YC YC. Analyzed the data: K-CJ X-JS A-PW. Contributed reagents/materials/analysis tools: K-CJ X-JS A-PW. Wrote the paper: K-CJ X-JS A-PW.